MITOTIC RESPIRATORY RHYTHMS IN SINGLE EGGS OF PSAMMECHINUS MILIARIS AND OF CIONA INTESTINALIS

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Using the reference (Cartesian) diver technique of Scholander, Claff and Sveinsson (1952), Scholander, Claff, Sveinsson and Scholander (1952) measured respiration of single eggs of marine animals, most of which had previously been studied on somewhat larger samples by Zeuthen (1949, 1950b, 1950c; Cartesian diver method, 1950a). All authors mentioned agree that the rhythm in mitotic division of the fertilized egg is accompanied by a faint respiratory rhythm. However, in the details there are certain discrepancies, especially concerning the reproducibility of the observed rhythm. The discrepancies shall be discussed in this paper which presents new data for single eggs of *Psammechinus miliaris* and of *Ciona intestinalis*. This paper reports all results obtained.

Method

1. Diver

The diver is the same as previously used by the author (1950a), only modified so as to work with one egg instead of a hundred, or a few hundred eggs. The increased sensitivity is obtained 1) by reducing as much as possible the dimensions of the diver and 2) by increasing the sensitivity of the manometer. The diver consists of a chamber and a stopper, both made from the same glass capillary of which two pieces are selected so that one fits tightly into the other. The diver's interior communicates with the exterior through capillary spaces left between stopper and chamber. These spaces permit free equilibration of pressure, and the stopper reduces diffusion. The procedure followed for making and filling this type of diver is illustrated in Figure 1 and is briefly described in the text for that figure. The diver has been drawn too short and thick. For correct dimensions, see inserts of Figures 4 and 6. In Figure 1 also other dimensions are misrepresented.

Before the diver can be filled for an actual experiment its weight must be adjusted so that it will float when a conveniently sized air bubble is introduced into the chamber. For this adjustment a suitable air bubble is pipetted into the newly made diver's chamber, using a Holter braking pipette (step 1 in Fig. 1). The stopper is loosely inserted and the diver is set afloat. It is, however, likely to be far from buoyant. From the rate of sinking or rising in water one soon learns to judge whether or not it shall be possible to regulate it to buoyancy. If prospects are good one first compresses or expands the air bubble by applying pres-

sure or suction to the system. If the diver is brought to float this way the necessary pressure change informs about how much smaller or bigger the air bubble should be chosen next time. Most often, however, it is necessary to remove or to add air and (or) glass. Air is removed or added from the bubble in the chamber, or the stopper's air volume is altered by letting the outer end of the stopper collapse or expand some. This can be done in a surprisingly regulated way by heating in a micro-flame mounted on the stage of the dissecting microscope. If the stopper's outer end is first drawn into a solid tail, the weight of the stopper can be altered by the addition to, or removal of, glass from this tail.

The air volume which finally makes the diver float is measured as the length of an air column in a calibrated Holter braking pipette which is used for all subsequent fillings of this individual diver. The air volume of the floating diver can also be derived from direct measurements of the linear dimensions of the air column in the floating diver.

It does require some skill to prepare these tiny and very fragile divers; many must be discarded. General rules: Always hold the diver with soft and flexible instruments. A piece of rubber, *e.g.*, a rubber stopper with a slit cut in the edge or across the one end, serves as excellent forceps. Watchmaker's forceps protected in the tip with flat pieces of rubber are useful. It is better to hold the divers with the fingers than with unprotected instruments. Capillarity should be made use of whenever possible. In air tiny pieces of glass adhere to wet glass rods and can thus be moved about without much risk of breakage.

2. Manometer

The diver's equilibrium pressure was adjusted and measured using a special "sensitive manometer" (Zeuthen, 1953b). The "manometer" is really a burette (Fig. 2 e, f, g, h), by means of which accurately measured volumes of fluid can be withdrawn from, or injected into, a closed air space (Fig. 2b extending from water surface in a through the ground joint c, the three-way tap d to the water surface e) in which regulated pressure changes are thereby created. The amount withdrawn is read by the movements of the bubble g in the burette. The diver floats in a small amount of alkaline medium in a pocket (a) open to the air space. The "manometer" can be adjusted to the nearest 1/25 mm. water pressure ($\backsim 4 \times 10^{-6}$ atm.) which is somewhat better than the sensitivity of the present diver.

At the beginning of the experiment the pressure in b is adjusted by mouth through n, m, l with d turned 90° relative to the position shown in the figure. With m closed, the manometer k is useful for the fine regulation of the initial flotation pressure in b; n is a CO_2-trap, l is an air brake. During actual measurements the three-way tap d is in the position indicated in the figure.

The volume of the "burette" from 0 cm. to 70 cm. is less than 1% of the volume of b, extending to e. Linear movements of g in the (very uniform) "burette" set up practically proportional pressure changes in b. The system can be reset when g has been moved across the scale. This introduces small changes in the value of h (equation below), which can, however, usually be neglected. The gas exchange is calculated from the formula

$$\frac{\mu l\ O_2}{\text{min.}} = \frac{x \cdot v \cdot V_D \cdot (B + h - e) \cdot 273}{(V + v) \cdot 10{,}300 \cdot (273 + t°)} = x \cdot K$$

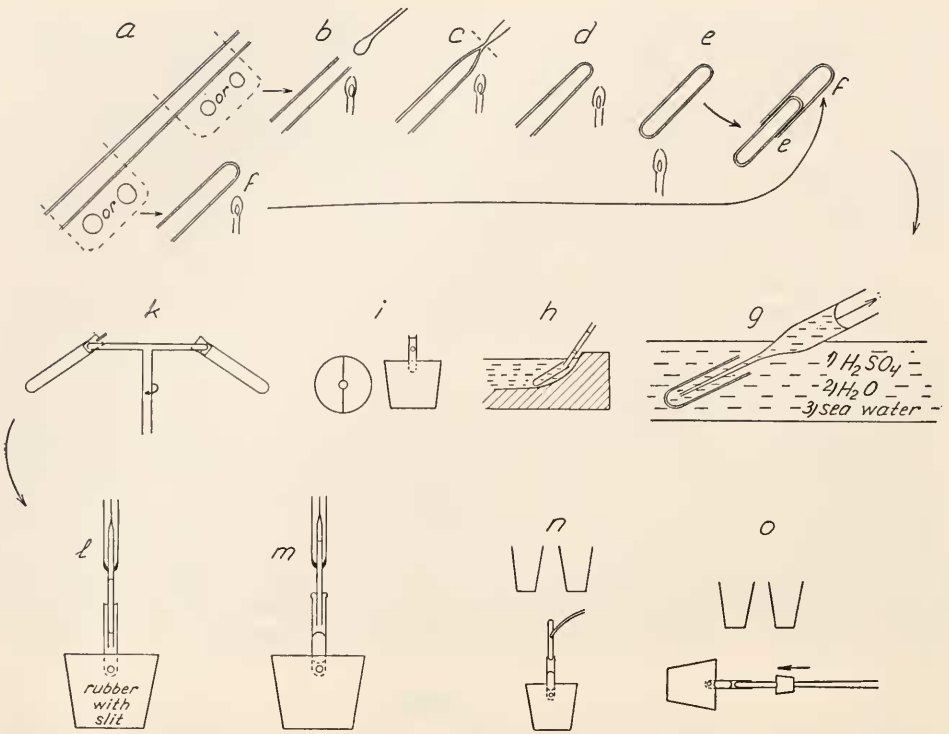

FIGURE 1. Method of making the diver. a. Pull a capillary (Thüringer glass) so as to obtain outside diameter *ca.* 0.2–0.25 mm; inside diameter = outside × 0.87–0.92. If divers made from a given capillary tend to become too heavy or too light, use more thin-walled or more thick-walled glass. Select two pieces of which I fits tightly when pushed a certain distance into II (use head lupe). The cross section of the capillary should be perfectly circular or it should deviate from circular in such a way that I and II can easily be oriented to fit each other. The alternatives are demonstrated. b–e. A micro-flame is mounted on the stage of a dissection microscope. The stopper is sealed—first at what is to be its inner end (upper right, b)—by making contact with a glass rod, pulling, cutting (square line in upper part of c indicates cut) and gently sealing the cut end. This way, a minimum of glass weight is used for sealing. Repeat in what is to be the outer end of the stopper (lower left of e), and seal the far end of the chamber (f). g. The chamber is rinsed in concentrated sulfuric acid and then successively in H_2O and in sea water, always by sucking large amounts of fluid into a pipette, which is introduced deep into the submerged chamber. h–m. The chamber is placed on the slanting side of a dish filled with sea water and the cell is dropped from a Holter braking-pipette into the open end of the diver chamber. The chamber is removed to a holder (i) which is an ordinary laboratory rubber stopper with a slit in it to hold the diver. The rubber stopper is selected to fit the jacket of a small hand centrifuge (k). The cell drops by its own weight to the bottom of the diver chamber or it is spun down with the centrifuge. *Ciona* eggs having a heavy coat of testa cells tend to stick to the glass. They can be pushed down with a small glass rod. The evaporation in the centrifuge is negligible ($< 5\%$). Air is introduced with a braking pipette (l) and, using a second pipette, the water in the upper end of the neck is replaced with alkali, isotonic with the sea water (m). For these operations the pipettes are mounted horizontally and the diver is moved vertically by the use of a special stand (*cf.* Holter, 1943). n. The rubber stopper holding the diver chamber is now placed under the dissection microscope so that the diver is in a vertical position. The stopper (for the diver) is brought to adhere by capillarity to the wet surface of a glass rod. While in the position indicated the glass rod is

in which x is the movement (in mm. per minute) of the bubble g in the "burette," v is the volume (μl) per mm. "burette," V is the volume of b extending to e (c. 100,000 μl; $v/V = 2 \times 10^{-5}$; in $(V + v)$ v can be skipped); V_D is the air volume of the floating diver. B is the barometric pressure in mm. H_2O (760 mm. Hg $\sim$ 10.300 mm. H_2O); h is the initial equilibrium pressure of diver (mm. H_2O) read on k as the difference in height of the menisci (with under-pressure, h negative); e is the vapor tension of water (or of n/10 NaOH and of 2% proteose-peptone) at $t°$ (mm. H_2O).

It should be noticed that x varies inversely with $(B + h - e)$. Therefore the sensitivity of the system should increase greatly at low pressures; and—as long as other factors are not limiting—it should approach infinity when h approaches negative values equal to $B - e$. The present experiments were carried out at total pressures $(B + h)$ varying from 323 to 578 mm. Hg.[1]

There are several possible ways of calibrating the manometer. The one here used is not claimed necessarily to be the best. The space b (from a to d, *cf.* Fig. 2) is determined by weighing with water. The pressure change induced in the whole system (b extending to e, m and meniscus in k), when g is moved from 0 to 70, is read on k, with the meniscus in the left branch adjusted to a defined level. This pressure change should be recalculated to what would have been observed if the space d, to m and k had not been included. The relative volumes of the systems: 1) b to e, m and k, 2) d to e, m and k, and 3) d to m and k (g in all cases in a stable position) are calculated from the pressure changes (read on k) induced for a defined linear movement of the left meniscus in k, when d is in the three positions defining the three spaces mentioned. The data obtained permit the calculation of the pressure change (mm. H_2O) induced in the space b, extending to e, for every mm. movement of g on the scale. In the above formula the calculated pressure change replaces $\frac{v}{V + v}$.

In one bath several "manometers" can be operated simultaneously more or less like Warburg manometers. In the present apparatus there are four manometers but for the present study only one was run at a time.

3. Bath, optical equipment

The bath was a 100-liter tank, on all sides, including the top, well insulated with cork plates. Holes were left open for stirring propeller, for manometer, for illumination from behind, and for observation. The bath was allowed to adjust itself to the room temperature and it varied in the range indicated in Table I, with maximum drifts in the course of an experiment of 0.1°–0.2°/hour.

The flotation vessel remained inserted into the bath for at least one hour before

[1] In this paper only rate curves are presented. It is only on very large-scale cumulative graphs that any scatter of the points can be demonstrated, and that the faint respiratory rhythm can be visually demonstrated. Lack of space prevents the publication of such curves.

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turned in a plane perpendicular to the paper so that the cross section of the stopper fits that of the chamber. Then the stopper is fitted into the chamber. Under the dissection microscope with the diver in a horizontal position, it can be pushed further in (o), using the rubber-protected tip of a watchmaker's forceps. The diver, thus filled, is dropped into a dish with alkaline flotation medium and transferred with a pipette to the flotation vessel (Fig. 2a).

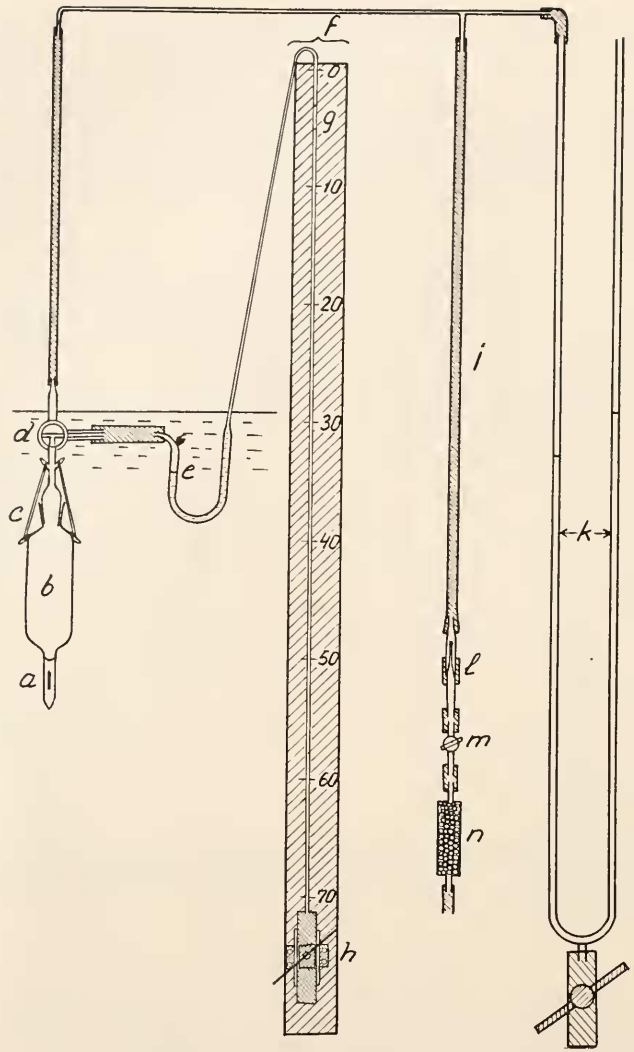

FIGURE 2. Manometer; *cf.* text.

the diver was introduced. This was done as gently as possible and a minimum of water was carried along. This way currents in the flotation medium were minimized during actual measurements, but they were not completely suppressed. It remains uncertain to which extent the initial adjustments in equilibrium pressure observed in most experiments (blanks and runs with egg) should be referred to currents in the flotation medium, to gradual establishment of diffusion steady-states inside the divers, and between flotation medium and gases in the space b, Figure 2, or to as yet unknown shortcomings of the present instrument.[2]

[2] This instrument, as also the 1950 diver, was designed for the demonstration of faint respiratory rhythms. For relative measurements it appears to be unsurpassed, but for absolute measurements it needs to be controlled for background drifts, and to be tested with objects which respire at known rates. Successful work on these lines was carried out by Mr. W. L. M. Geilenkirchen working in this laboratory. The paper will appear in a Dutch journal.

Horizontal microscopes (12.5 × 2.5) with ocular scales are firmly mounted in front of the bath, one for each manometer. The diver's equilibrium pressure is read by "turning point" determinations. The diver is kept floating at about the same level (± 0.1 mm.) throughout the experiment. Whenever a reading is due the manometer is set so that the diver rises slowly. At the same time the respiration makes the diver heavier, and it therefore turns on some level. The manometer, when first set, is not again touched before the diver has turned and the pressure has been read. Figure 3 demonstrates some trials with a respiration diver (1.35 μl), which is subjected to varying initial underpressures. Curves I

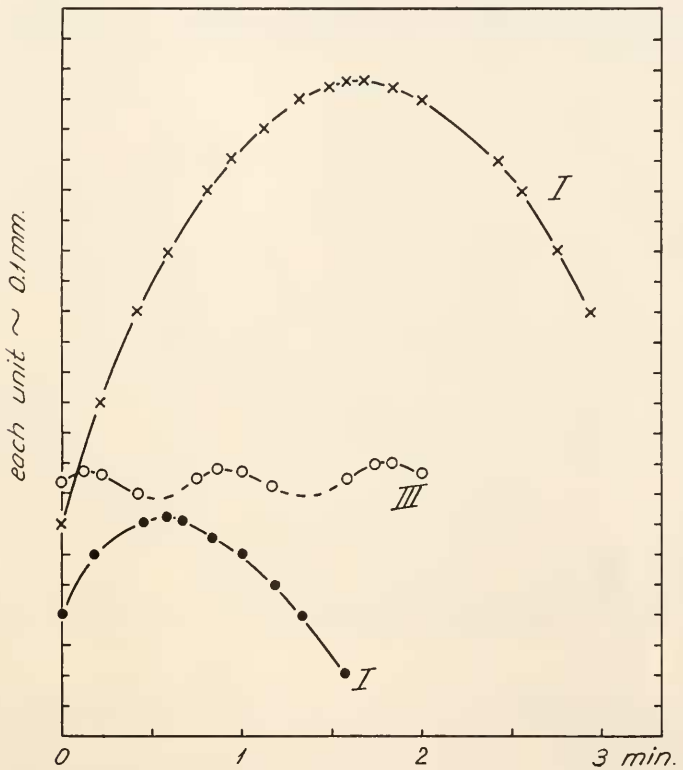

FIGURE 3. Rates of movement of a respiration diver which was subjected to varying initial under-pressures.

represent two cases in which the diver was given relatively low initial pressure and high initial rate of movement. The changing positions are plotted as a function of time. Curve III shows that the level on which the diver turns can be reproduced to about 0.03 mm. and also that the diver can be kept continuously moving up and down within a narrow zone. The results would have been even better if the observer had not had to make notes. Incidentally, one is not forced to accept a reading if the diver happens not to turn on the right level. For the present investigation the measuring periods were about 300 seconds. It is claimed that the turning point can be determined within, say, ± 3 seconds or about ± 1% of the time be-

tween successive readings or better. Close inspection of the data underlying the curves of Figure 3 indicates that about 0.005 mm. displacement of the diver was enough for the observer to decide about the direction of the diver's movement. In the optical field this corresponds to 0.15-mm. displacements. Suitable reference marks on the diver are points which in the beam of light appear like little stars. If such a point happens to form a continuation of one of the lines of the ocular scale the decision is about whether the line extended with the "star" tends to bend up or down in the optical field.

4. *Material*

The eggs of *Ciona intestinalis,* fertilized and unfertilized, were used. The testa cells were not removed; they have been found to make up for 20–30% of the res-

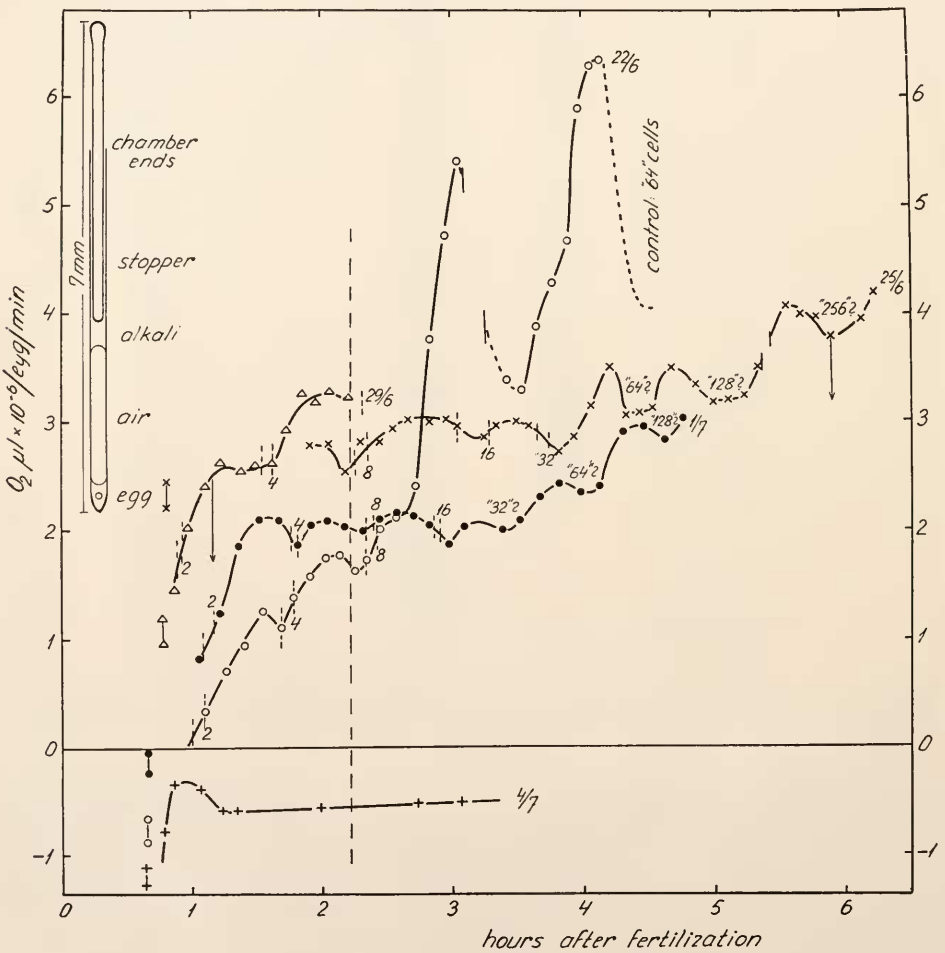

FIGURE 4. Four experiments on single eggs of *Psammechinus miliaris,* with one control experiment (4/7); *cf.* Table I and the text.

piration of the unfertilized egg (Holter and Zeuthen, 1944) and less in the case of the fertilized egg. The eggs were thoroughly washed before use. Another object used was the fertilized egg of *Psammechinus miliaris,* Z-form. The jelly was removed with a pipette, the fertilization membrane was retained. Both animals are from the littoral zone and the experiments were run in sea water, salinity 20–25‰. The divers were floating in 22‰ NaCl, made up to n/5 with isotonic NaOH; no Na-taurocholate was added.

Experiments

A. *Psammechinus miliaris.* Figure 4 shows four experiments with single eggs of *Psammechinus.* The same diver was used throughout, and an experiment with empty diver is also reported (4/7). The ordinate shows the rate of oxygen uptake per egg per minute, and the abscissa indicates hours after fertilization.

Table I

Oxygen consumption in single eggs of Psammechinus miliaris. Data pertaining to Figures 4–6

		$B+h$ (mm. Hg)	t_1^0	t_2^0	O_2/min., µl (135 min.)	Vol./egg, µl	µl O_2/µl egg/min.
I	22/6	340	16.9	17.3	2.2×10^{-6}	6.25×10^{-4}	3.5×10^{-3}
	25/6	450	18.2	17.7	3.2×10^{-6}	4.9×10^{-4}	6.5×10^{-3}
	29/6	533	19.6	19.8	3.8×10^{-6}	5.25×10^{-4}	7.2×10^{-3}
	1/7	418	18.6	19.3	2.6×10^{-6}	4.8×10^{-4}	5.4×10^{-3}
	4/7	344	21.1	—	—	—	—
II	20/6	538	16.9	17.4	2.6×10^{-6}	5.0×10^{-4}	5.2×10^{-3}
	24/6	523	17.5	17.5	2.1×10^{-6}	4.75×10^{-4}	4.4×10^{-3}
	7/7	578	19.9	20.0	3.2×10^{-6}	—	—
III	4/7	362	—	—	—	—	—
Aver.		466	18.5		2.8×10^{-6}	5.2×10^{-4}	5.4×10^{-3}

I, fertilized eggs, Figure 4. II, fertilized eggs, Figure 5. III, control experiment, Figures 4 and 5.

Two points connected with a vertical line indicate the time when the diver was initially brought to float in the flotation medium. The position of these pairs of points on the ordinate is in all cases arbitrary. Due to space limitation two curves (three in Fig. 6) have been displaced on the ordinate to the extent indicated by the length of the arrows. Gaps on these curves (and on other curves presented) indicate resetting of the manometer. Important characteristics of the 5 experiments are given in Table I. The two temperatures are at (or near) the beginning and the end of each experiment.

The diver, shown with typical filling in the insert of Figure 4, has a gas volume (V_D) of 0.063–0.065 µl, determined slightly differently, in the 5 experiments. The internal cross sectional area (A) of the diver capillary was calculated from several diameters and V_D was calculated from the formula $A\cdot(l-2r)+4/3\pi r^3$ in which l is the length of the air column from meniscus to meniscus and $2r$ is the (average) diameter of the diver capillary; l was measured on the floating diver. In the graphs

pairs of vertical stippled lines across a curve indicate the time when a division was beginning and when it was completed. The number of cells resulting from a division is indicated. After the 16-cell stage (32-cell stage on 25/6) accurate observations were no longer possible. The numbers placed by the time of later minima on the curves represent guesses. We actually know (*cf.* Zeuthen, 1951, 1953a) that "64," "128" and "256" cell stages do not exist. The cell numbers are in all cases lower than indicated. The long stippled line (2 h. 15 min.) shows the time when the absolute rates reported in Table I were measured as the distance from curve to curve (4/7) for the control experiment. Egg volumes were calculated from the measured diameter of the experimental egg itself, or from the average diameter of several control eggs.

The position of the control curve (4/7) indicates that probably in all cases there is some diffusion of gas into the diver from the surroundings. Unfortunately, the diver was broken before another control was run, and this causes some uncertainty as to the absolute rates measured. However, Borei (1948) for both *Psammechinus* forms ("Z" and "S") found an average rate of O_2 uptake of 3.1×10^{-6} μl/egg/min. (18°, 120 min. after fert., egg size 4.43×10^{-4} μl). The results here reported check well with this. Inspection of the rate curve 22/6 for slope indicates that we have reason to suspect the value (Table I) for this experiment to be too low.

The egg used on 25/6 stayed in the diver until the mesenchyme-blastula stage; the eggs used on 22/6 and on 1/7 were removed immediately after the experiment and not observed further. The egg studied on 29/6 stopped development by the time of the third division. By this time the experiment had to be discontinued because the stirring motor set up disturbing vibrations; two hours later the four blastomeres of this egg had fused into two. By this time most of the control eggs were blastulae, but a number had developed into monsters of the same type as the one in the diver.

Figure 5 presents two more experiments with single *Psammechinus* eggs, both showing asynchrony between divisions of the two first blastomeres. The egg used on 20/6 showed a mild degree of asynchrony, the second division being delayed 3–4 minutes in the one blastomere. In the following division cycle this delay could again be observed; 7 cells were directly counted—there may perhaps have been 8. In this experiment optical conditions were poor. Possible division activity was noted at times indicated (?). On the following morning this egg was a mesenchyme blastula (while still in the diver); the controls were gastrulating. The blastomeres in the egg studied on 24/6 showed pronounced asynchrony of divisions. The number of cells observed at any time is indicated, optical conditions were good. Both experiments (20/6, and especially 24/6) are inconclusive as to respiratory cycling, but they may indicate that pathological conditions which bring about asynchrony of division may at the same time increase the amplitude of the respiratory cycles above normal.

Such a situation, if correct, suggests that physiological interaction between neighboring cells which are in different phases of mitosis might result in increased cycling amplitude. This hypothesis was put to a preliminary test, with negative result, in the experiment of 7/7. For this experiment a good batch of eggs was divided into two lots and fertilized with a time interval of 17 minutes, which roughly equals half of one mitotic cycle. The jelly, and in most eggs also the

fertilization membrane, was removed shortly after the fertilization using a narrow pipette. From the lot first fertilized, 92 eggs in the 2-cell stage were selected, 44 without membrane and 48 with. From the second lot 92 eggs were isolated in the 2-cell stage, all without membrane. All 184 eggs were thoroughly mixed together and introduced into a diver (gas space: 9.8 μl). The resulting respiration curve (7/7) is indicative of simple interference between two groups of eggs, each of which shows respiratory cycling of about the magnitude shown in the typical curves of Figure 4. Thus, this experiment supplied no evidence of physiological

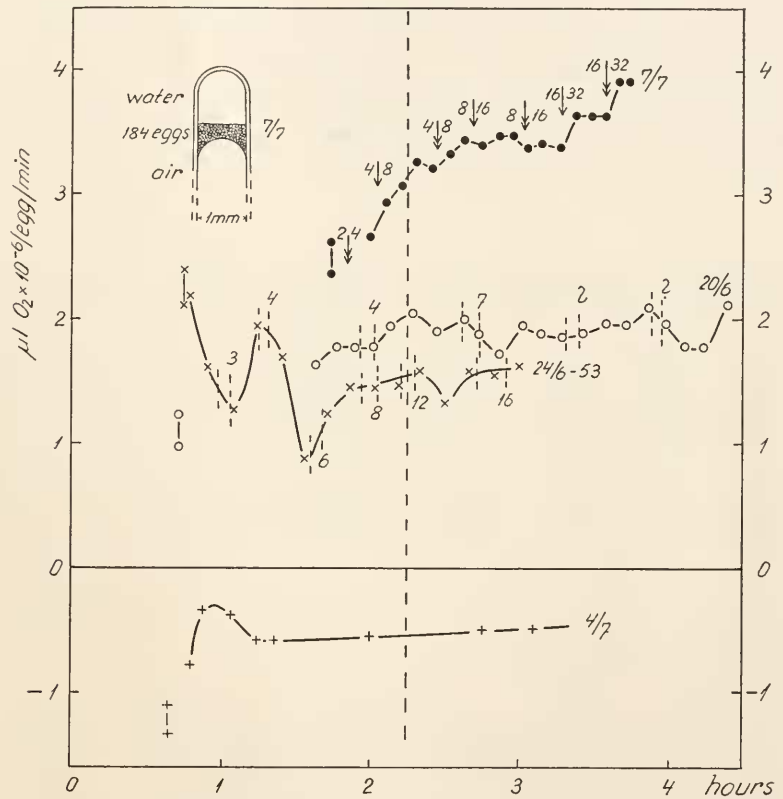

FIGURE 5. Two deviating experiments (20/6, 24/6) on single *Psammechinus miliaris* eggs. One experiment (7/7) with 184 eggs fertilized in two separate lots (92 + 92 eggs) showing absence of physiological interaction between the two groups.

interactions between two groups of cells which are much out of phase. In Figure 5 single and double arrows indicate the approximate division times of control eggs belonging to the two separate lots.

The absolute respiration rates measured two hours 15 minutes after fertilization are given in Table I. The experiment 4/7 serves as base line for the experiments on 20/6 and 24/6. The base line for the experiment 7/7 is the 0-line.

B. *Ciona intestinalis.* The diver used for these experiments looked much the same as the one used in *A;* however, all dimensions were slightly larger. V_D

measured as under A averaged 0.112 μl (variation 0.110–0.115, for Experiment 8/6 a mistake was made when the dimensions were measured—the calibration found on earlier days was accepted). In Figure 6 rate of oxygen uptake is plotted on an arbitrary time scale. Time for beginning and for end of each division is indicated with stippled vertical lines, and the resulting number of cells is indicated; (?) indicates poor conditions of observation. The experiments on 11/6 and 5/6 are for unfertilized eggs, and the experiments on 3/6, 6/6 (2 exps.) and 8/6 are on fertilized eggs which all showed beautiful development in the diver (not observed beyond the time of experiment).

The two control experiments on 10/6 were made with the same diver as used for all *Ciona* experiments, but with certain changes introduced by accident. After completion of the experiment on 8/6 some of the outer end of the diver chamber broke off. Therefore the diver became buoyant with a reduced value of V_D (0.060 μl instead of 0.112) and the diffusion barrier set up in the diver's neck after introduction of the stopper became shorter, *i.e.*, probably less efficient. Be-

TABLE II

Oxygen consumption in single eggs of Ciona intestinalis. Data pertaining to Figure 6

		$B+h$ (mm. Hg)	t_1^0	t_2^0	O_2/min., μl (1 hour)	Vol./egg, μl	μl O_2/μl egg/min.
	3/6	473	18.32	18.50	1.05×10^{-5}	2.4×10^{-3}	4.35×10^{-3}
	6/6(1)	455	18.52	18.55	1.03×10^{-5}	—	—
I	6/6(2)	506	18.55	18.75	1.22×10^{-5}	2.35×10^{-3}	4.7×10^{-3}
	8/6	463	17.88	18.22	1.14×10^{-5}	2.3×10^{-3}	4.65×10^{-3}
II	5/6	570	18.21	18.35	0.83×10^{-5}	—	—
	11/6	323	—	19.83	0.75×10^{-5}	2.0×10^{-3}	3.75×10^{-3}
III	10/6	460	—	—	—	—	—

I, Fertilized eggs. II, Unfertilized eggs. III, Control exp.

fore the accidental loss of glass the stopper fitted the diver chamber over a distance of 3.75 mm. This distance was reduced to about 3.1 mm. The diver thus modified was floating at a pressure not too different from that used in the biological experiments (*cf.* Table II) and the readings represented by the hollow circles were obtained. Then the diver was removed from the vessel and the stopper was pulled about one mm. back in order still further to reduce the diffusion barrier in the diver's neck. The diver was set afloat again and the readings represented by black circles were obtained. The (small) difference in level between the control curves and the 0-line is accepted as representing a maximum of diffusion of gas into this diver in all experiments.

In Table II the respiration intensity for each egg is measured (at time one hour) by the distance between experimental and control curves. Also the cell volume is given, based on direct measurements of two cell diameters or on an average diameter for at least four control eggs.

The respiration intensity here measured for single eggs in early cleavage stages

(1.0–1.2 $\times 10^{-5}$ μl/egg/min.) compares well with results reported in 1944 for single *Ciona* eggs by Holter and Zeuthen (*ca.* 1 $\times 10^{-5}$ μl/egg/min. at 22.5°), also when temperature differences and possible volume differences between eggs are taken into account. The present experiments were run at a 4° lower temperature than used in 1944 but with eggs which on an average seem to have been slightly bigger than in 1944. Also for the unfertilized eggs the over-all rates check with the earlier report.

Discussion

Out of a total of 10 experiments on single dividing marine eggs (4 *Ciona* eggs, 6 *Psammechinus* eggs) 6 showed respiratory cycling of much the same kind (*Psammechinus* 25/6, 29/6, 1/7, Ciona 3/6, 6/6 (2), 8/6). A seventh (*Psammechinus* 22/6) showed the same kind of cycling during divisions 2 and 3. The atypical experiments are the following: *Psammechinus:* one experiment (24/6) showed strong respiratory variation before the 8-cell stage, and one (22/6) considerable variations between 16- and 64-cell stages; one experiment (20/6) was inconclusive. One *Ciona* experiment (6/6 (1)) showed considerable early variations, but was interrupted early.

Concerning the deviating experiments it should be stressed that sometimes (*cf.* control 4/7) readings within the first hour or so are uncertain. The reasons are imperfectly understood. However, the always possible presence of such technical difficulties places considerable doubt on all non-reproducible deviations from the general findings, and the author is therefore unable to decide whether the results on 6/6 (1) and on 24/6 should be referred to errors in the technique or to the respiring cells themselves. On 22/6 the curve for the early part of the experiment seems to indicate stability of the system. However, by about the time of the 4th division the respiratory rate suddenly increased steeply and the respiration ended up so intense that the scale was used up after four more readings. Then the manometer was reset, *i.e.*, the pressure in the system was lowered and the diver was equilibrated with the indicator air bubble back in the upper end of the scale. The two first readings after this showed low rate of respiration but then the rate accelerated much the same way as before. Again, one is unable to decide between the egg or deficiencies in the apparatus as the cause. A leakage developing in stop-cock d, Figure 2, and re-developing after the stop-cock was operated with the resetting of the manometer, *might* perhaps result in effects as those recorded.

For *Ciona* two experiments on unfertilized eggs are available. One of the two (11/6) might perhaps be fitted with a cycling curve, but the author feels that the least committing interpretation is absence of cycling. There are independent reasons for not depending too much on the experiment on 11/6. It was run at such a low pressure (*cf.* Table II) that the manometer had to be reset too often, as demonstrated by the many gaps on the curve.

Had all runs on *Ciona* eggs, fertilized and unfertilized, been fitted with smooth, non-cycling curves, we should evidently have found more scattering around the curves for dividing, fertilized eggs than for non-dividing, unfertilized eggs. This suggests that the respiration in fertilized eggs is subject to variations not present in unfertilized eggs, and where possible the curves are drawn to suggest that these variations are rhythmic and timed with the mitoses.

It seems doubtful whether the present techniques—as also the one of Scholander *et al.* (1952)—is reliable enough accurately to record the respiratory variations in every run, and many more control experiments with unfertilized eggs would have been necessary if we were to predict the frequency with which an experiment with fertilized egg would fail to demonstrate a rhythm actually present. It is strongly emphasized that in this situation the degree of reproducibility of the rhythmic phenomenon itself remains our main control. The type of cycling respiration which is demonstrated throughout the curves for the experiments on 25/6, 29/6, 1/7, in part of the curve 22/6 (all for *Psammechinus*) and on 3/6, 6/6 (2) and 8/6 (for *Ciona*) the author considers as reproducible as can be expected in view of the technical difficulties involved. They largely confirm results he has previously obtained on single frog eggs and in mass runs on different marine eggs. All deviating observations, discussed in detail above, he considers interesting but quite inconclusive.

Scholander *et al.* (1952) published a material of 19 runs on four different species of single dividing eggs which were studied for rhythmic respiration. As far as can be gathered from the description of Scholander *et al.* a few eggs show definite respiratory cycling, one even very strong cycling; 10–11 eggs show definite cycling with an amplitude much the same as found by the present author. However, the authors state that it almost vanishes after the two or three first divisions. Five experiments are inconclusive as to cycling, or it is concluded that there is no cycling. Of these 5, the 4 were with Urechis eggs which show the lowest rate of respiration of the eggs studied. The most constant cycling was in single Dendraster eggs which on an average respire more intensely than any of the other three species of eggs studied. One exceptional case of an egg (*Strongylocentrotus purpuratus*) showing abnormally intense cycling is suggestive. However, this egg was definitely sick. It developed very slowly and in an asynchronous way (*cf.* Fig. 5 of this paper).

On the basis of this material Scholander *et al.* concluded that respiratory cycling is observed, but (p. 197): "We must emphasize, however, that cell division in individual cells very often takes place without any demonstrable cycling. Quite regularly, therefore, the energy requirements for the different phases are apparently fitted nicely together within the limits of a steady constant flow of oxidative processes." In the absence of experiments with objects which can be expected to show non-rhythmic respiration the reproducibility of any type of rhythmicity is the only control, and to the present author the stated absence of such reproducibility in the material of Scholander *et al.* does not invite conclusions as the above quoted. First, proof is required that the method will reproduce constant rhythmicity, if present, in every run. Substantiated doubts whether the method of Scholander *et al.* will do this have been expressed on a previous occasion (Zeuthen, 1953a). To the present author all evidence, viewed together, indicates as good reproducibility as for technical reasons can be expected of the phenomenon of respiratory cycling in cleaving eggs.

Scholander *et al.* also conclude (p. 197) that: "In our experiments the cycling is always strongly damped. It almost vanishes after the two or three first divisions." They further write (p. 197): "Zeuthen (1949) has found that later on cycling increases very markedly. Even if it can be demonstrated that this late cycling also occurs in single eggs, there will still be a minimum of cycling after

the second or third division, and hence there can be no simple correlation between the wave amplitude and the steadily increasing number of cell divisions." The views previously expressed by Zeuthen (1949) are these (p. 316): Up to the stage of division 4–5 in *Psammechinus miliaris,* division 6–7 in *Dendraster excentricus* and in *Strongylocentrotus franciscanus,* limit unknown for frog and Urechis the extra respiration is a function of the constant mass of the embryo rather than of the number of cells dividing. But after this stage has been passed the extra respiration becomes more closely a function of the number of cells dividing than of the mass of the embryo. The "extra respiration" of each division was

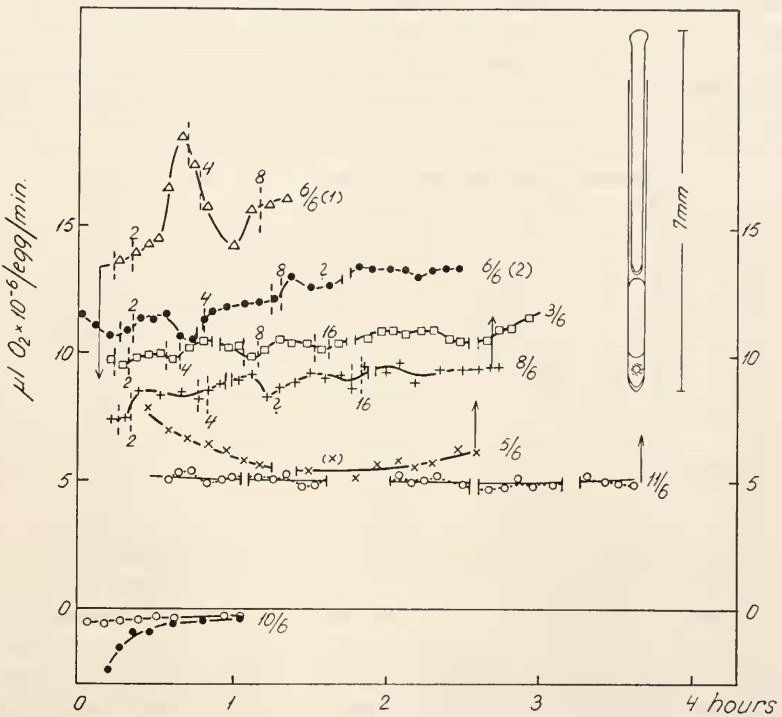

FIGURE 6. Four experiments (3/6, 6/6 (1), 6/6 (2), 8/6) on fertilized dividing *Ciona* eggs, two (5/6, 11/6) on unfertilized eggs, and two control runs (10/6) without eggs.

defined as the small areas, from one minimum on the respiration curve to the next, between the respiration curve and a smooth auxiliary line drawn to touch all minima.

The first half of the above statement, about constancy of the respiratory cycling in the early divisions, is confirmed by the present experiments. The author finds no evidence of decreased cycling after the first 2–3 divisions. On the other hand, the increased cycling with the late divisions is only slight. However, it was commented already in 1949 that in this latter respect different eggs behave differently. In the situation now established it should be pointed out that within the single egg asynchrony of divisions begins with divisions 4–5 and tends to become pro-

gressively worse later, even though a statistical rhythm persists throughout cleavage (*cf.* Zeuthen, 1951; Holter and Zeuthen, 1955). Slightly increased respiratory cycling by the time when asynchrony sets in is therefore indicative of definitely increased cycling in the single blastomeres.

Further discussing a point raised by Scholander *et al.*, Claff (1953) expressed doubts about one of Zeuthen's main summary points (1951) which is (p. 66): "In the segmenting egg the oxygen consumption follows a rhythm which throughout segmentation correlates with the mitoses." However, he quotes evidence only from 1949 and 1950, not from the paper from which the summary point is taken. This evidence (Zeuthen, 1951; Holter and Zeuthen, 1955) is the following: Mitotic rhythmicity occurring throughout 10 successive cycles was demonstrated in batches from single females of *Psammechinus microtuberculatus.* It was stated that increasing rates of metabolism occurred whenever a considerable majority of the cells were in mitotic stages characterized by having nuclei with nuclear membranes. For single eggs it was stated that asynchrony between divisions progresses from

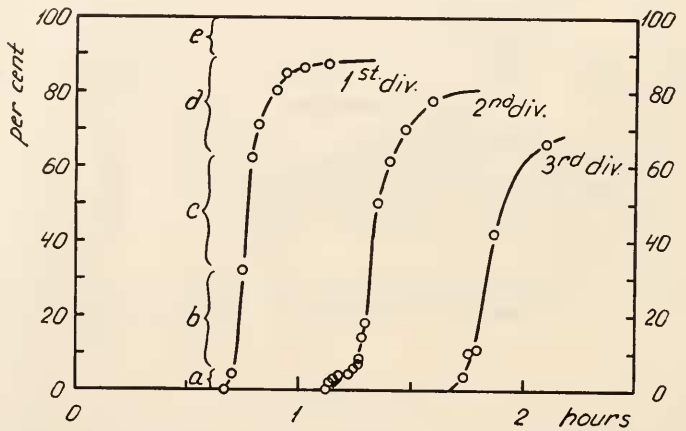

FIGURE 7. Unequal distribution of division frequencies in the 1953 Kristineberg material.

16-cell stage on, but a manifest statistical mitotic rhythm persists throughout cleavage. Scott and Fox (1952), quoted by Claff in support of his views, do not disprove the presence of a statistical division rhythm throughout cleavage in batches of Arbacia eggs, and they did not measure respiration. Actually, their findings can very well be interpreted to agree with Zeuthen's data (1951) demonstrating a statistical division rhythm.

Nevertheless, it is of course true that the degree of synchronization in batches cannot be expected always to be equally good. During the summer 1953 the author had intended to do mass runs on *Psammechinus miliaris,* Kristineberg Z-form, and to study the influence of inhibitors on the respiratory rhythmicity. However, respiratory cycling was only occasionally observed. This was nothing new or surprising because it happens whenever the egg material shows poor synchronization of cleavage. Usually this difficulty can be overcome by selection of suitable females, but not this year.

The degree of synchronization of divisions was then investigated in the follow-

ing way. From a batch of fertilized eggs (one female) showing more than 90% fertilization, with nicely lifted membranes, 100 fertilized eggs were selected with a Holter braking pipette within three minutes after fertilization. The curves of Figure 7 give the relation between time after fertilization and per cent of cells entering the first, the second, and the third division. The three curves demonstrate progressively more and more unequal distribution of division frequencies, with 30% being excessively delayed already in the first division; the number of eggs which at all divide decreases with each new division. The eggs entering the first division were separated into five groups (a–e incl.), a representing those which first enter division, d those which cleave with most delay. Group e (seven cells) was discarded. These eggs divided directly into four cells. Whereas group a developed nicely to swimming blastulae, groups b, c, d stopped development at earlier stages and the earlier, the more first division was delayed. Six hours after fertilization all eggs in group a (four eggs) were young blastulae (swimming next morning); in group b (28 eggs) after six hours fourteen were young blastulae, six less advanced "morulae" and eight were eight-cells. Next morning thirteen were swimming blastulae, the others had not advanced further. Six hours after fertilization, group c (31 eggs) were "morulae" and did not develop further; group d (30 eggs) all remained in division stages with anything from one to eight cells. This type of experiment was repeated several times with comparable results, but the one here reported was the most striking. These observations present very interesting problems but could not be followed up because our time at the zoological station was up. It should be stated that the spawning season was not yet over; also that exceptional climatic conditions this year (extreme heat with surface temperatures approaching 25°) influenced the eggs strongly. Everybody complained about "poor material."

The curves of Figure 7 demonstrate heterogeneities in the 1953 Kristineberg material which unquestionably represent extreme tendencies which may or may not exist in all batches of sea urchin eggs. The 1947 *Psammechinus* material used at Kristineberg by the author in his first experiments on respiratory rhythms in dividing eggs was definitely better than the 1953 eggs. Nevertheless, already in 1947 it was found useful (*cf.* Zeuthen, 1950a) to select small batches of eggs for experiments as those individuals in a population (one female) which first enter first division. This secured for the 1947 respiration experiments relatively synchronously developing small groups of eggs. In later experiments on other sea urchins and on Urechis no such selection was made for the reason that representative small samples of whole batches from "good females" (picked on basis of quality of membrane lifting and of percentage fertilization) showed reproducible respiratory cycling throughout cleavage. The author accepts as likely that the eggs of those species for which the nicest respiratory cycling was observed in mass runs may also have been those which showed the highest degree of synchronization of development in whole batches. On this basis he deems *Dendraster excentricus* (1949) and *Urechis caupo* (1949, 1950c) the best material with which he has worked. Still, it was for the Naples *Psammechinus microtuberculatus* that respiratory cycling and mitotic activity was demonstrated to correlate throughout cleavage (Zeuthen, 1951).

In the following the situation demonstrated for the 1953 Kristineberg material

shall be considered extreme and atypical. We shall accept that usually (*cf.* Blum and Price, 1950 for *Arbacia punctulata*) there is a normal distribution in time of the frequencies with which eggs in a population from one female enter every new division and we shall further, for the sake of simplicity, assume that all eggs in a small population show identical respiratory variations only with some displacement of the curves in time. How much, then, shall the excursions on the respiration curves found for single eggs be blurred in records for the whole population?

In Figure 8, I, the curve for *Psammechinus*, July first, was accepted as common for all eggs in a small population of 5. This curve was re-plotted five times (a) at time intervals of three minutes, which means that there is a difference in time of twelve minutes between the fastest and the slowest egg. A full cycle lasts anywhere from twenty-eight to forty minutes. The five curves are then mixed (b, heavy curve) to give the respiration curve for the population for comparison with the curve for a single egg. In Figure 8, IIb, the same procedure is repeated, only with the curves plotted five minutes apart (twenty minutes between fastest and slowest). In the cases of Figure 8, I, II, the divisions are equally, not normally, distributed in time, *cf.* distribution diagrams; a more natural situation is constructed in Figure 8, III. In this case the two heavy curves from Figure 8, Ib, IIb, are mixed with one curve for a single egg. The resulting heavy curve for this population is indicated. In this population there is reasonably good approximation to a normal distribution of identical stages in time (*cf.* diagram to the right in 8, III). The striking fact is that although in Figure 8, III, the fastest and the slowest cells are separated by a time corresponding to 5/7 of the shortest cycles, all respiratory variations for the single eggs are recorded in the population, but of course with some damping of the wave amplitude, especially in the case of the shortest cycles. In Figure 8, III, the abscissa is chosen so that zero corresponds to zero consumption. The respiratory variations observed by the author for single frog eggs (1946) and later for early divisions in small populations of marine eggs are very much alike when shape and amplitude (*ca.* 5% of total rate) are concerned. Linderstrøm-Lang's calculations (1946) demonstrated that the records for a single frog egg may have been subject to some damping caused by diffusion in this fairly big system. The true variations may have occurred with an amplitude of 7–11%. In the case of the marine eggs the diffusion factor can hardly have been of significance (Zeuthen, 1950c), but as demonstrated in this paper there may have been damping of about the same order of magnitude (slightly different from one group of experiments to another) as in the frog egg experiments, however due to incomplete synchronization. It is now considered that the curves here published for single marine eggs (Figs. 4 and 6) represent very close approximations to actual intracellular variations in the rate of oxidation, typical probably of many types of dividing egg cells.

Figures 4 and 6 show that it is no longer possible to describe the respiration of dividing eggs as following a sine curve. Clearly, in *Psammechinus miliaris* and in *Ciona intestinalis* there is a rather sharp dip in the rate of oxygen consumption reaching a minimum around the time of every new division, especially divisions 2–5 inclusive, and rapidly again increasing after the division. With later divisions the sharp dips change into broad valleys. The respiratory rate tends to remain on a high level for some time prior to the next dip, especially so in the early

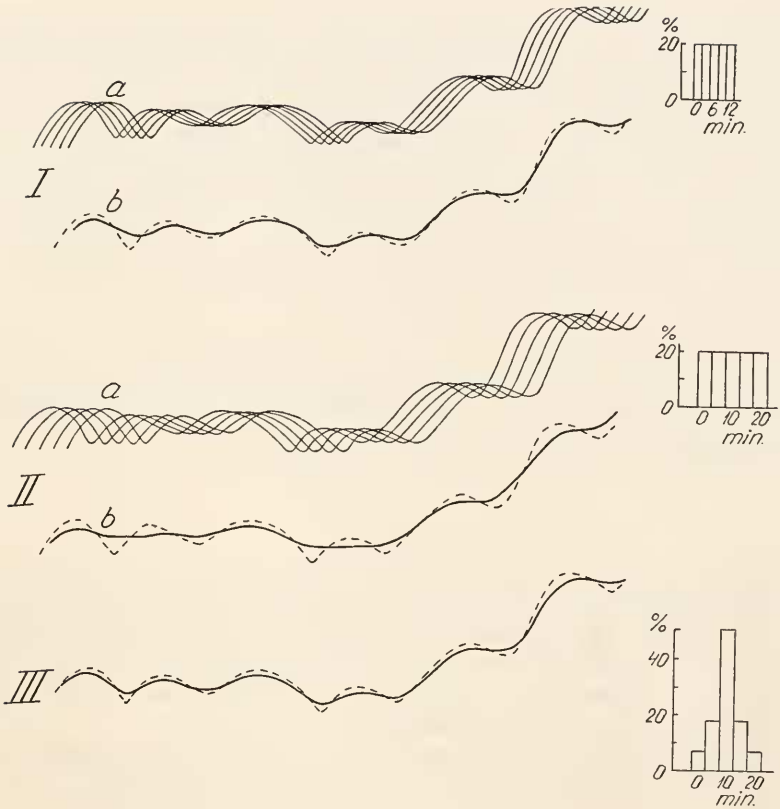

FIGURE 8. Construction of average curves for populations (heavy curves Ib, IIb, III) of eggs which are assumed to respire according to identical curves, only with some displacement in time. The dotted curves in Ib, IIb, and III are identical with the one curve replotted 15 times in Ia, IIa. The diagrams to the left in the figure show the frequencies with which the cells in the populations I, II and III enter every new division.

divisions. The cell division sometimes begins already when the respiration is decreasing, more often when it is minimum or already increasing. This confirms most of the previous results of the author (frog (1946) sea urchins (1946, 1949, 1950b, 1951) the ciliate *Tetrahymena piriformis* (1953b)) and of Scholander *et al.* (1952) (sea urchins, *Urechis*). In sea urchins, the respiratory rate (*cf.* Zeuthen, 1951; Holter and Zeuthen, 1955) seems to be minimum around late telophase, it increases sharply during inter- and prophase, remains level around mid-mitosis and drops again in late mitotic phases. Sometimes, however, the division has been observed to occur early in the phase of decreasing respiratory rate. This was for a few (not all) runs on several hundreds of *Urechis* eggs (1950c); however, in this case conditions for optical observations were admittedly much poorer than in runs with single eggs. If confirmed, this finding warns us that the respiratory cycling may appear to be more closely associated with other mitotic events than with the cytoplasmic fission (*cf,* discussion in Zeuthen, 1951; Holter and Zeuthen, 1955).

This view is corroborated by the following facts: A single fertilized frog egg which altogether failed to divide showed normal respiratory cycling (1946) and colchicin-treated fertilized eggs of *Psammechinus microtuberculatus* (1951) which did not divide showed respiratory cycling with increased amplitude and extended period.

The observed respiratory rhythm shows inverse relation to the state of development of the Mitotic Apparatus (M.A.) of Mazia and Dan (1952). The M.A. is a structure which is tied together by S-S-bridges. This may be highly important, suggesting some connection between SH-groups and respiratory cycling. However, for several reasons it is too early to make any deductions from this observation.

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Summary

1. In single fertilized marine eggs (*Psammechinus miliaris, Ciona intestinalis*) the rate of respiration shows a temporary decrease (5–10%) and reaches a minimum every time the egg divides. The findings were reproduced in 6½ experiments out of 10. The method is not yet well enough controlled to permit definite statements as to whether or not deviating experiments should be referred to insufficient technique or to the eggs themselves.

2. The respiratory rhythm demonstrated resembles the one previously observed in the frog egg, in the *Urechis* egg and in several echinoderm eggs.

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